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(71) Applicants: REPROGENESIS, INC. [US/US]; 21 Erie Street, Cambridge, MA 02139 (US). CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 300 Longwood Avenue, Boston, MA 02115 (US).

(72) Inventors: BORLAND, Kermit, M.; 43 Park Street, Shrewsbury, MA 01545 (US). ZHOU, Tao; 223 Forest Park, Durham, NH 03824 (US). NELSON, Gordon, P.; 939 Mt. Hope Street, N. Attleboro, MA 02760 (US). ATALA, Anthony; 74 Westerly Road, Weston, MA 02193 (US).

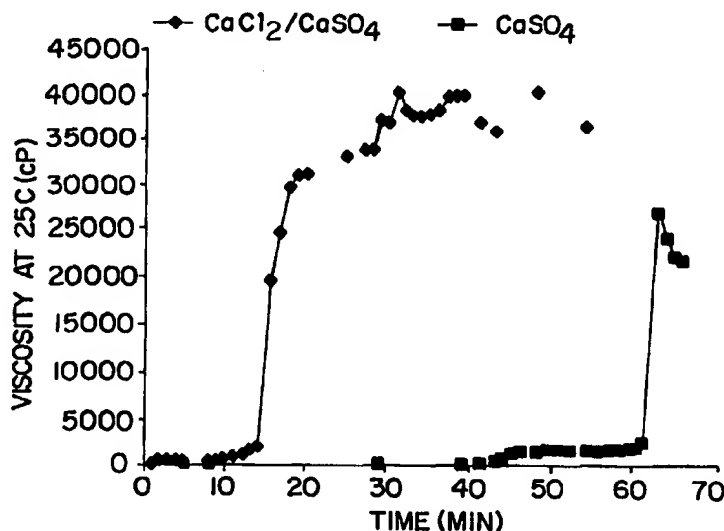
(74) Agents: POSORSKE, Laurence, H. et al.; Baker & Botts, L.L.P., The Warner, 1299 Pennsylvania Avenue, N.W., Washington, DC 20004 (US).

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(54) Title: IMPROVED HYDROGEL COMPOSITION



(57) Abstract

This invention provides compositions for use in implanting cells into an animal comprising cells (which may be dissociated cells and/or cell aggregates); a biodegradable, biocompatible polymer which forms a hydrogel upon cross-linking by multivalent ions; a soluble salt of a multivalent ion; and a sparingly soluble salt of a multivalent ion, these components being combined into a mixture which forms a partially hardened, injectable hydrogel in which the cells are uniformly suspended, the consistency of the mixture being suitable for implanting the partially hardened hydrogel mixture into the animal, where the implanted, partially hardened hydrogel forms *in situ* a fully hardened hydrogel containing the cells. Preferably, the mixture also contains a biocompatible sequestant which competes with the biocompatible polymer for binding of the multivalent cross-linking ion. The invention also provides methods for implanting cells in an animal using the composition.

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IMPROVED HYDROGEL COMPOSITION

BACKGROUND OF THE INVENTION

Field of the Invention

- 5 The present invention is generally in the area of creating new tissues using polysaccharide hydrogel-cell compositions.

Review of Related Art

- Craniofacial contour deformities, whether traumatic, congenital, or aesthetic, currently require invasive surgical techniques for correction.
- 10 Furthermore, deformities requiring augmentation often necessitate the use of alloplastic prostheses which suffer from problems of infection and extrusion. Techniques of tissue engineering employing biocompatible polymer scaffolds hold promise as a means of creating alternatives to prosthetic materials currently used in craniomaxillofacial surgery, as well as
- 15 formation of organ equivalents to replace diseased, defective, or injured tissues. Tissue engineering involves the morphogenesis of new tissues from constructs formed of isolated cells and biocompatible polymers. Cells can be adhered onto a polymeric matrix and implanted to form a cartilaginous structure. This can be accomplished, as described in U.S. Patent No.
- 20 5,041,138 to Vacanti, et al., by shaping of the matrix prior to implantation to form a desired anatomical structure and surgical implantation of the shaped matrix.

- A simple method of delivering additional autogenous cartilage or bone to the craniofacial skeleton that is site specific and of controlled
- 25 dimensions would reduce surgical trauma and eliminate the need for allografts or alloplastic prostheses. If one could transplant by means of injection or simple application to the desired site, and cause to engraft large numbers of isolated cells, one could augment the craniofacial osseocartilaginous skeleton with autogenous tissue but without extensive

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surgery. Furthermore, successfully implanting isolated cells would create the potential for tissue culture augmentation of the cells.

Transplantation via injection which can engraft large numbers of isolated cells, and augment the craniofacial osteo-cartilaginous skeleton with autogenous tissue, but without extensive surgery is clearly preferable. However, it has been shown that dissociated cells injected subcutaneously or within areas of the body such as the peritoneum have not been successful. Cells are relatively quickly removed, presumably by phagocytosis and cell death. Extending tissue engineering techniques to a system whereby the cell-polymer constructs are delivered less invasively and retained at the delivery site has been investigated to expand the applicability of tissue engineering to fields such as plastic surgery. Mixtures of dissociated cells and biocompatible polymers in the form of hydrogels have been used to form cellular tissues and cartilaginous structures including non-cellular material which will degrade and be removed to leave tissue or cartilage that is histologically and chemically the same as naturally produced tissue or cartilage. Slowly polymerizing, biocompatible, biodegradable hydrogels have been demonstrated to be useful as a means of delivering large numbers of isolated cells into a patient to create an organ equivalent or tissue such as cartilage. The gels promote engraftment and provide three dimensional templates for new cell growth. The resulting tissue is similar in composition and histology to naturally occurring tissue.

Unlike the use of solid polymer systems to create a cell-polymer construct, a liquid support matrix that polymerizes to a gel is more easily shaped and molded for custom reconstruction or augmentation. Additionally, a liquid polymer system can potentially be used for injectable delivery, which would be much less invasive than open implantation. Calcium alginate gels have been proposed as a means of delivering large numbers of isolated chondrocytes to promote engraftation and cartilage formation. These initial studies were extended in International Patent

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Publication No. WO 94/25080 to the formulating of slowly polymerizing calcium alginate gels and to the use of these gels to deliver large numbers of chondrocytes by means of injection, for the purpose of generating new cartilage.

5 The endoscopic treatment of vesicoureteral reflux was first introduced in 1981 when polytetrafluoroethylene (Teflon) was injected in the subureteral region of a patient. More than a decade later the search persists for an ideal injectable substance. Particle migration to distant organs raised concerns regarding the use of polytetrafluoroethylene paste. The high rate
10 of re-treatment necessary due to implant volume loss has limited the usefulness of collagen. The ideal implant material should be nonmigratory, nonantigenic and delivered endoscopically, and should conserve its volume. Toward this goal, long-term studies to determine the effect of chondrocytes *in vitro* and *in vivo* determined that alginate, a biodegradable polymer,
15 embedded with chondrocytes, would serve as a synthetic substrate for the injectable delivery and maintenance of cartilage architecture *in vivo*, and this system has been tested for the treatment of vesicoureteral reflux in a porcine model. However, the behavior of the system was less than satisfactory.

Injectable compositions containing cells have been produced by
20 suspending suitable cells in medium M199, and mixing the cell suspension with an equal part of 2% sodium alginate solution, then adding solid calcium sulfate powder to initiate cross-linking of the alginate to form a hydrogel. Such a composition typically will contain 1% sodium alginate in a hydrogel with insoluble calcium sulfate in an amount of about 200 mg per ml of
25 suspension. A small amount of calcium chloride may be provided by the cell suspension, but usually less than 0.1 mg per ml in the final suspension. Experience indicates that such suspensions have a latent period on the order of an hour, followed by a rapid increase in viscosity to produce a relatively hard, even brittle gel within half an hour of the viscosity increase.

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However, the consistency of hydrogel-cell suspensions described above is not totally satisfactory for use in injection into patients in need thereof. In some cases, the hydrogel-cell suspension hardens before it can be injected into the patient. Such gels are useful for preparing preformed
5 structures for implantation into patients to repair structural defects of known shape, but require extensive surgery to open up a cavity of sufficient size to receive the preformed structure.

On the other hand, some hydrogel-cell suspensions maintain low viscosity until injection into the desired location in the body, but such
10 suspensions assume the shape of the existing cavity or space in the patient. Such gels cannot be used where the defect is absence of a defined structure. Thus, there remains a need for a hydrogel-cell suspension with sufficient consistency to maintain its shape when implanted in the patient, but which remains injectable to minimize the extent of surgery necessary for
15 implantation. In particular, shortcomings in the present injectable cartilage formulation that would disallow its use in clinical trials include (a) insufficient viscosity to divide the tissue plane and not extravasate upon injection; (b) extended time required prior to achieving high enough viscosity to attempt injection; and (c) inconsistent performance between lots
20 due to poor distribution of components during formulation.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a cell suspension which may be injected into a surgical site, but will assume and maintain a desired shape after injection. In particular, it is an object of this invention to provide
25 a cell suspension (a) having a consistency similar to POLYTEF teflon paste at injection; (b) achieving this consistency immediately upon mixing, and (c) retaining that consistency for a long period of time to allow proper application.

It is another object of this invention to provide a cell-containing
30 hydrogel which will not migrate from the desired site, will retain its shape

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against gravity, and can be applied to a specific area by several means, including pumping through an injection cannula. These and other objects may be met by one or more of the following embodiments.

In one embodiment, this invention provides a method for implanting
5 cells into an animal, the method comprising (1) forming a mixture containing cells, which may be dissociated cells and/or cell aggregates; a biodegradable, biocompatible polymer which forms a hydrogel upon cross-linking by multivalent ions; a soluble salt of a multivalent ion; and a sparingly soluble salt of a multivalent ion, such that the mixture forms a partially hardened,
10 injectable hydrogel in which the cells are uniformly suspended; and (2) implanting the partially hardened hydrogel mixture into the animal, wherein the implanted partially hardened hydrogel forms a fully hardened hydrogel containing the cells *in situ*. Preferably, the biocompatible polymer is alginate. Preferably, the multivalent ions are metal cations other than
15 magnesium; more preferably the metal cations are calcium. Optionally, the mixture also contains a sequestrant which competes with alginate for binding calcium ions, preferably an anionic sequestrant such as phosphate anion. In a particularly preferred embodiment, the soluble salt is calcium chloride and the sparingly soluble salt comprises a cation selected from the group
20 consisting of copper, calcium, aluminum, magnesium, strontium, barium, tin, and di-, tri- or tetra-functional organic cations; and an anion selected from the group consisting of low molecular weight dicarboxylic acids, sulfate ions, carbonate ions, phosphate ions and citrate ions. More preferably, the sparingly soluble salt is calcium sulfate and the mixture contains calcium
25 chloride/calcium sulfate in ratio of from 0.001 to 100, preferably in ratio of from 0.0075 to 1.0 by weight. In an exemplary embodiment, the mixture contains at least 0.5 % alginate by weight and at least about 0.001g calcium chloride per gram of alginate. Suitable cells are selected from the group consisting of chondrocytes and other cells that form cartilage, osteoblasts

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and other cells that form bone, muscle cells, fibroblasts, and organ cells. In preferred embodiment, the cells are chondrocytes isolated from the patient.

In another embodiment, this invention provides a method for treating anatomical defects in an animal in need of treatment thereof comprising
5 preparing a suspension of cells uniformly dispersed in a biodegradable, biocompatible hydrogel solution which is partially hardened, and implanting the suspension into the animal at the site of the defect, wherein the hydrogel solution fully hardens after implantation. Preferably, the partially hardened hydrogel has a viscosity at 25°C of at least 15,000 cps or the suspension of
10 dissociated cells in the partially hardened hydrogel is suitable for injection through a 23-gauge cannula at a rate of 10 ml/min. without substantially damaging the cells.

In yet another embodiment, this invention provides a composition for implanting cells into an animal comprising a hydrogel containing aggregated
15 cells or dissociated cells, such as chondrocytes and other cells that form cartilage, osteoblasts and other cells that form bone, muscle cells, fibroblasts, and organ cells; a biocompatible polymer which forms a hydrogel upon cross-linking by multivalent cations; a highly soluble ionic salt of a first multivalent cation; and a sparingly soluble salt of a second
20 multivalent cation, the composition forming a partially hardened hydrogel at room temperature which is suitable for injection into an animal and which, after injection, will fully harden *in vivo*. The first and second multivalent cations may be the same or different and are selected from the group consisting of copper, calcium, aluminum, magnesium, strontium, barium, tin,
25 and di-, tri- or tetra-functional organic cations; and an anion selected from the group consisting of low molecular weight dicarboxylic acids, sulfate ions and carbonate ions. Typically, the partially hardened hydrogel is suitable for injection through a 23-gauge cannula at a rate of at least 10 ml/min. without reducing viability of the cells by more than 25%. Preferably, the
30 composition contains as the biocompatible polymer, alginate at a

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concentration of at least about 0.5%, as the highly soluble salt, calcium chloride at a concentration of 1.5 mg/mL, and/or as the sparingly soluble salt, calcium sulfate at a concentration of 15 mg/mL. Where the highly soluble salt is calcium chloride and the sparingly soluble salt is calcium sulfate, the ratio for calcium chloride:calcium sulfate is preferably from 0.001 to 100, more preferably from 0.0075 to 1.0 by weight. Preferably, the compositions contains a sequestrant which competes for binding of the second multivalent cation with alginate. More preferably, the sequestrant is phosphate anion. Suitable cells may be selected from the group consisting of chondrocytes, osteoblasts, muscle cells, fibroblasts, and organ cells; preferably, the cells are chondrocytes.

The present invention provides a readily available source of cross-linking cations, generally in the form of calcium chloride, at a level sufficient only to initiate and partially cross-link a biocompatible anionic polymer (e.g., alginate) to a thick paste consistency, preferably in approximately 15 minutes. More preferably, alginate is present in an amount sufficient to incorporate all water in the formulation into the loosely cross-linked gel (typically greater than about 0.5% alginate is present in such injectable cell suspensions). Such a formulation will set up quickly for prompt use in an operating theater, while maintaining good "injectable" consistency for an extended period of time to allow completion of procedures. The consistency of this partially cross-linked hydrogel may be maintained in excess of 24 hours. Furthermore, the suspension has a thick consistency which more easily divides tissues when injected and is less subject to extravasation from the injection site.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the time course of gel formation at 25°C for alginate cross-linked with calcium sulfate powder and calcium chloride/calcium sulfate, respectively.

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Figure 2 shows the time course of gel formation at 37°C for alginate cross-linked with calcium sulfate powder and calcium chloride/calcium sulfate, respectively.

Figure 3 shows the time course of gel formation at 5°C for alginate cross-linked with calcium sulfate powder and calcium chloride/calcium sulfate, respectively.

DETAILED DESCRIPTION OF THE INVENTION

Cell/polymer systems which form hydrogel compositions for tissue engineering include suspensions of cells such as chondrocytes in alginate solution to which calcium salts are added to initiate hydrogel formation. Typical systems include chondrocyte-calcium alginate solutions created by vortexing an isolated cell suspension with sodium alginate solution (e.g., in 0.1M K_2HPO_4 , 0.135 M NaCl, pH 7.4) to yield a cellular density of 20×10^6 cells/ml (a cellular density of approximately 50 percent of that of native articular bovine cartilage) in a 1.0% alginate solution. The chondrocyte-sodium alginate suspension can be stored on ice at 4°C until use. Prior to injection, 0.2 gm of sterilized $CaSO_4$ powder is added to each milliliter of the cold chondrocyte-alginate solution.

Studies on the gel formation properties of formulations using calcium sulfate as the source of cross-linking ion (see, e.g., Example 1) show that the formulation can gel in as little as 16 minutes at human body temperature, but does not gel readily at room temperature. When the temperature of the alginate/cell suspension is raised to the level necessary to initiate gel formation within a reasonable time (i.e., on the order of minutes rather than hours in the operating room), a window of only several minutes exists during which the consistency of the composition is suitable for injection. Failure of the formulation to gel at room temperature necessitates the use of heating equipment for incubation of the formulation prior to injection. Additionally, mixing and injecting problems occur due to the formation of gel around

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CaSO₄ particles which blocks the transfer of Ca⁺² through the solution, and further blocks injection through a syringe cannula by forming clots of alginate-encapsulated CaSO₄. Therefore, a filter must be attached to the syringe in order to screen out lumps created by more rapidly gelling regions
5 in the suspension. These characteristics increase the complexity in the formula's clinical application and make it difficult to achieve any lot-to-lot consistency in delivery and gelling properties.

To avoid the requirement for heating the mixture plus adding a filter on the injection syringe, a new formula has been developed for injectable
10 hydrogels used in tissue engineering. This formula provides for accelerated gel formation and prolonged injectability of the gel suspension, to obtain a consistent performance.

The method of this invention involves formation of hydrogel by multivalent cation cross-linking of acidic biopolymers in which the
15 availability of the multivalent cation is controlled by dissolution of a sparingly soluble salt to replenish soluble cation as the soluble cation is absorbed in cross-links. A portion of the multivalent cation is provided by a fully soluble salt and is freely available ("fast" cross-linking ion), while another portion of the multivalent cation necessary for full cross-linking is
20 provided in a sparingly soluble salt ("slow" cross-linking ion). These components are preferably provided in a composition that also contains a soluble buffering system for the multivalent cation, usually phosphate when the cation is a di- or tri-valent metal ion. Phosphate competes with alginate for the metal, so that cross-linking of the alginate in the composition is
25 forestalled by competition between alginate and phosphate anions for the limited amount of available cation as the components of the composition are mixed.

In an exemplary embodiment, the biocompatible acidic biopolymer alginate is cross-linked by a minor portion of fully-soluble calcium chloride
30 and a major amount of the much-less-soluble calcium sulfate. The

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composition from which the hydrogel is formed contains phosphate anion in relatively high concentration to buffer the soluble calcium ion concentration and forestall complete cross-linking of the alginate. Once the composition is injected at the desired site, phosphate anion is diluted out by diffusion into the surrounding biological fluids, and the concentration of soluble calcium ion increases locally. As the soluble calcium ion concentration increases, more calcium cross-links are formed in the alginate, and the hydrogel hardens. Similar effects may be obtained for analogous metal ions by buffering with phosphate anion or other similar systems, e.g., biologically compatible sequestrants, which buffer soluble cation concentration in an analogous manner.

Sources of Cells.

This technique can be used to provide multiple cell types, including genetically altered cells, within a three-dimensional scaffolding for the efficient transfer of large number of cells and the promotion of transplant engraftment for the purpose of creating a new tissue or tissue equivalent. It can also be used for immunoprotection of cell transplants while a new tissue or tissue equivalent is growing by excluding the host immune system.

Examples of cells which can be implanted as described herein include chondrocytes and other cells that form cartilage, osteoblasts and other cells that form bone, muscle cells, fibroblasts, and organ cells, and also includes expanded populations of isolated stem cells. As used herein, "organ cells" includes hepatocytes, islet cells, cells of intestinal origin, cells derived from the kidney, and other cells acting primarily to synthesize and secrete, or to metabolize materials.

The cells to be encased in the injectable hydrogel can be obtained directly from a donor, from cell culture of cells from a donor, or from established cell culture lines. In the preferred embodiments, cells are obtained directly from a donor, washed and implanted directly in combination with the polymeric material. Cellular material may be

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dissociated single cells, minced tissue (i.e., clumps of aggregated cells) or cell aggregates generated *in vitro* from dissociated cells. The cells may be cultured using techniques known to those skilled in the art of tissue culture.

Cell persistence and viability after injection can be assessed using scanning electron microscopy, histology, and quantitative assessment with radioisotopes. The function of the implanted cells can be determined using a combination of the above-techniques and functional assays. For example, in the case of hepatocytes, *in vivo* liver function studies can be performed by placing a cannula into the recipient's common bile duct. Bile can then be collected in increments. Bile pigments can be analyzed by high pressure liquid chromatography looking for underivatized tetrapyrroles or by thin layer chromatography after being converted to azodipyrroles by reaction with diazotized azodipyrroles ethylanthranilate either with or without treatment with P-glucuronidase. Diconjugated and monoconjugated bilirubin can also be determined by thin layer chromatography after alkalinemethanolysis of conjugated bile pigments. In general, as the number of functioning transplanted hepatocytes increases, the levels of conjugated bilirubin will increase. Simple liver function tests can also be done on blood samples, such as albumin production.

Analogous organ function studies can be conducted using techniques known to those skilled in the art, as required to determine the extent of cell function after implantation. For example, islet cells of the pancreas may be delivered in a similar fashion to that specifically used to implant hepatocytes, to achieve glucose regulation by appropriate secretion of insulin to cure diabetes. Other endocrine tissues can also be implanted. Studies using labeled glucose as well as studies using protein assays can be performed to quantitate cell mass on the polymer scaffolds. These studies of cell mass can then be correlated with cell functional studies to determine what the appropriate cell mass is. In the case of chondrocytes, function is defined as synthesis of cartilage matrix components (e.g., proteoglycans,

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collagens) which can provide appropriate structural support for the surrounding attached tissues.

Precursor cells of chondrocytes, or cells derived from pluripotent stem cells which have the capability of differentiating into chondrocytes, can also be used in place of the chondrocytes. Examples are fibroblasts or mesenchymal stem cells which differentiate to form chondrocytes. As described herein, the term "chondrocytes" includes such chondrocyte precursor cells.

Polymer

The polymeric material which is mixed with cells for implantation into the body should form a hydrogel. A hydrogel is defined as a substance formed when an organic polymer (natural or synthetic) with charged side groups is cross-linked via ionic bonds to create a three-dimensional open--lattice structure which entraps water molecules to form a gel. Examples of materials which can be used to form a hydrogel include polysaccharides such as carrageenan or alginate, the latter being the preferred biopolymer for formation of cell-containing hydrogels of this invention.

Alginate is a copolymer of stretches of polymannuronate and polyguluronate obtained from seaweed. Suitable alginate is available from commercial sources. Individual alginate preparations will have a determinable capacity for binding calcium which is a function of the mannuronate:guluronate (M/G) ratio. Effects resulting from varying the ratio between mannuronate and guluronate residues are described in U.S. Patent No. 4,950,600, incorporated herein by reference.

The method of this invention for controlling gelation characteristics and final gel properties using multiple cation sources to supply cross-linking cations, where the cation sources are chosen for disparate solubilities, could also apply to other polymers, such as the biopolymer κ -carrageenan.

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Cross-linking Agent

The water soluble polymer with charged side groups is cross-linked by reacting the polymer with an aqueous solution/suspension containing multivalent ions of the opposite charge, either multivalent cations if the polymer has acidic side groups or multivalent anions if the polymer has basic side groups. The preferred cations for cross-linking of the polymers with acidic side groups to form a hydrogel are divalent and trivalent cations such as copper, calcium, aluminum, magnesium, strontium, barium, and tin, although di-, tri- or tetra-functional organic cations such as alkylammonium salts, e.g., $R_3N^+ - \text{VVV} - \text{NR}_3$ can also be used. Aqueous solutions of the salts of these cations are added to the polymers to form soft, highly swollen hydrogels and membranes. The higher the concentration of cation, or the higher the valence, the greater the degree of cross-linking of the polymer. Concentrations from as low as 0.005 M have been demonstrated to cross-link these polymers. Higher concentrations are limited by the solubility of the salt.

The preferred multivalent ion for use with the preferred biopolymer (alginate) is calcium. The method of this invention is described herein using calcium and calcium salts as representative cross-linkers. Other multi-valent ions which perform similarly are, of course, within the contemplation of this invention.

To rapidly generate sufficient consistency to divide tissue planes upon injection and to prevent extravasation while avoiding the problems of blocked injection cannulas, this invention provides a hydrogel forming composition containing two sources of cross-linking polyvalent cation: a minor amount of one cation source which provides fully available cation and a major amount of another cation source which provides slow release of the cross-linking ion as the gel is formed. The first cation source is a highly water soluble salt - i.e., solubility of at least 1g per 100 ml, preferably at least 30g/100ml, more preferably at least 60g/100ml. Particularly preferred

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is a calcium salt such as calcium chloride. The second cation source is a sparingly soluble salt - i.e., less than 1g/100ml, preferably less than 0.5g/100ml, more preferably less than 0.3g/100ml. Particularly preferred is a calcium salt such as calcium sulfate. Any two salts of multivalent cations
5 having similar water solubilities to calcium chloride and calcium sulfate, respectively, are also suitable for use in this invention. While either salt alone may provide sufficient cross-linking cation to produce a fully hardened alginate hydrogel, use of two salts spreads the cross-linking action over time, thereby expanding the time window during which a partially hardened
10 hydrogel is of suitable consistency for injection through a cannula.

Calcium chloride is one preferred "fast" Ca^{+2} source to speed up the alginate gelling because of its high solubility. Other calcium salts of equivalent solubility work in a similar fashion. Experiments were run to determine if CaCl_2 alone could work. A concentration range of 1.0 to 6.6
15 mg/ml was tried, and it was found that CaCl_2 alone was not suitable to cross-link the cell-containing hydrogel, since the alginate gelled up immediately at the interface of alginate and CaCl_2 solutions when the concentration of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ is equal to or higher than 3.3 mg/ml, but would not gel up at all when equal to or lower than 2.7 mg/ml when alginate was used at a final
20 concentration of 1.5% (w/v). Depending on alginate concentration and desired gel strength, CaCl_2 may range from 0.5mg/ml to 3.0 mg/ml.

The amount of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ required to react stoichiometrically with sodium alginate is 0.309 mg per mg of alginate. A typical known calcium sulfate cross-linked formulation uses 20 mg $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ per mg of sodium
25 alginate, or approximately 65 times the amount required. The solubilities of calcium sulfate dihydrate ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) in cold and hot (100°C) water are 2.41 and 2.22 mg/ml, respectively. Mixing and injecting problems occur however, due to the formation of gel around CaSO_4 particles which blocks the transfer of Ca^{+2} through the solution and further blocks injection through
30 a syringe cannula by forming clots of alginate-encapsulated CaSO_4 .

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Proportions are preferred which produce a homogeneous gel of acceptable consistency in required time. The preferred ratio of $\text{CaSO}_4/\text{CaCl}_2$ depends on the polymer (the M/G ratio for alginate), the required time for gelation, required texture strength, etc.. As an example, for
5 a sodium alginate which can bind 0.309 g of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ per g of alginate, the ratio of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}/\text{CaSO}_4 \cdot 2\text{H}_2\text{O}/\text{alginate}$ can be 1:10:10 when certain amount of phosphate presents in the formulation.

For example, suitable compositions may have proportions which produce a gel of acceptable consistency in approximately 14 minutes at room
10 temperature. The ratio of $\text{CaSO}_4/\text{CaCl}_2$ which provides about 3 mg calcium chloride and 30 mg of calcium sulfate per 30 mg of alginate is suitable for use with alginate which binds 0.309 g of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ per g of sodium alginate. The stoichiometry for alginate with a different calcium binding capacity can be readily determined by one skilled in the art, and similar mole
15 ratios of sparingly soluble and freely soluble salts can be calculated to determine suitable amounts of these alternative salts. M/G ratio of the alginate determines the Ca-binding capacity. Preferably, the relative amounts of alginate, fully soluble salt and sparingly soluble salt, and optionally soluble sequestrant, will be such that the mixture forms a gel
20 within 20 minutes that will hold its shape against the force of gravity, but may be injected without clogging the injection canula for at least 4 hours. (These times are for incubation of the mixture at room temperature.)

As discussed above, formulations using only slowly soluble cation sources produce a partitioning of components into alginate-encapsulated
25 clods of undissolved salt and a more liquid portion that could be collected and injected. Such a product is poorly reproducible in consistency, cannot be well-characterized, well-controlled, or altered to give the final formulation a range of properties. Use of only rapidly soluble cation sources can produce partitioning as well, if the cation cannot be distributed
30 throughout the mixture, as portions of fully cross-linked gel will be produced

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and are not easily injected. Addition of both a sparingly soluble and a freely available multivalent cation source may circumvent these problems. However, the inventors have found that mixing the alginate with two cation sources in the presence of a cation-sequestering anion which provides an excess of cation binding capacity produces a final formulation that also demonstrates an improvement over existing gels: notably homogeneous mixing and production of a consistent suspension, with controlled and uniform properties throughout, that is completely injectable.

Consequently, the hydrogel forming composition according to this invention optionally contains a component which will buffer the amount of cross-linking cation available to the biopolymer. The buffering component is usually an anion sequestrant such as phosphate anion. Rapid and complete cross-linking of the alginate is prevented by the PO_4 anions in the formula competing with alginate in binding of Ca^{+2} , which greatly reduces the gelation speed. Phosphate anion is typically present at about 0.1M, but the phosphate concentration may be varied from 0.03M to 0.3M to control gelling time. Other biologically compatible sequestrants which will compete with alginate for calcium, and thus prevent formation of alginate encapsulated salt particles in a similar manner, are also contemplated by this invention.

An excess of cation binding capability and competition between anions and alginate for that available cation permits a complete distribution of the available cation throughout the suspension while simultaneously allowing a uniform distribution of all other components (e.g., cells, low solubility cation, gel) of the formulation. The product is homogenous throughout, is produced consistently, can be characterized by conventional testing protocols, is entirely available for application without product loss and can be adjusted to impart desired properties (e.g. viscosity, gel strength, gelation time, etc). This uniformity in mixing is demonstrated in different

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mixing formats, such as vortexing or hand mixing in test tubes, magnetic and mechanical stirrers, within mixing syringes, etc.

Mixing protocols

Substantial variation is permitted in the order of addition for the components which react to form the cell-containing hydrogel. Generally, the cells are added before the hydrogel reaches its partially hardened consistency. Typically this means that the cells are introduced into a premixture containing either the alginate or one or more of the cation sources before the cation sources and the alginate are combined. Usually cells are first mixed with alginate, optionally including the sequestering anion, and then the cation sources are added in one or more steps. In one example, chondrocytes, or other suitable cells, are harvested, grown to confluence, passaged as needed, then mixed with a biodegradable liquid polymer such as alginate which is designed to solidify at a controlled rate upon subsequent mixing with multivalent ionic salts. Variations in the levels of individual components, or conditions of mixing and incubation can be modified to a) control the consistency of the material as injected; b) control the time required for attaining an injectable consistency; c) and controlling the properties of the final gel *in vivo* to accommodate particular requirements of the transplanted cells for engraftment and function or of the receiving tissue site for appropriate texture and dimensional retention.

Levels of individual components, either singly or in combination, can be modified to alter different properties of the formulation both before and after application so as to accommodate particular requirements a) for injection and application, b) for individual cell viability or successful engraftment and creation of required properties and function of the final gel and replacement tissue, or c) for the manufacture, distribution, and application to patients of the formulation. For example, injection of a cell/gel formulation into compact tissues (e.g. muscle, submucosa) requires a high viscosity to prevent extravasation of material, while application to

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achieve a large surface area distribution (peritoneal injection) or to coat preformed structural elements (vascular grafts, stents, polymer scaffolds, etc.) requires a solution of lower viscosity. Alterations of viscosity can be achieved by a number of mechanism either singly or in concert, such as (1) selection of the viscosity of the raw material (e.g. low, medium, or high viscosity alginate); (2) concentration of gel (e.g. a range of 0.3% to 3.0% alginate can be used to achieve a broad range of gel viscosities); (3) amount of highly soluble multivalent cation source to control degree of partial cross-linking; or (4) level of anions provided to compete with alginate for cation binding. Successful engraftment of different cell types, or the nature of the desired replacement tissue to be created may require alterations of the formulations components. For example, creation of a firm, compact tissue (e.g. liver, cartilage) requires a gel of higher polymer chain length or concentration (e.g. >1.5% alginate, high guluronic content alginate), while simply coating a surface such as bone surface augmentation using a gel/osteoclast formulation is more successful with a lower concentration of polymer.

Controlling the duration of injectability and properties of a formulation prior to injection would aid the manufacture, distribution, and application of gel/cell therapies. The working time of the final formulation, or the time required for materials to setup to an injectable consistency, can be controlled through multiple methods, such as by the amount of highly soluble cation provided and/or the level of anion provided to compete for cation binding, in addition to temperature regulation. For example duration of an injectable consistency can be controlled through temperature so as to allow a working time of minutes (34-38°C) or beyond one month (4-8°C). Temperature plays a very important role in the gel formation speed. Upon increasing the temperature, as will occur, for instance, when the material is injected into tissue having a temperature of 37°C, the material will proceed to harden into a fully cross-linked hydrogel. As shown in Example 1, at

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CaSO₄·2H₂O level of 0.1 x (i.e. 20 mg/ml in a formulated batch) the partially hardened gel could set up in 32 minute at body temperature and remain injectable for at least 90 minutes. This best case scenario of CaSO₄ at 37 °C still requires too much time to reach injectable consistency, and is both more
5 complicated and more inconsistent than desired.

Cation sources can be chosen to control gelling and gel properties, and also combined to control degradation. The composition according to this invention, having both a fast and slow source of cross-linking agent, can provide both more rapid viscosity increase, and a longer time before
10 becoming fully cross-linked compared to the formulation with calcium sulfate alone. In a particularly preferred mode, the mixture which forms partially hardened hydrogel will also include a significant amount of phosphate anion. Typically, the phosphate concentration will be approximately 0.1 molar.

15 Increasing the concentration of alginate is also helpful for the gel to hold more water, resulting in a thicker paste and a stronger gel, and also in accelerating the gel formation. Preferred alginate level is at least 0.75%, more preferably between 1.5% and 2.5% and up to 3.0%, in the final cell-containing suspension which forms the hydrogel.

20 In a preferred embodiment of this invention, the acidic biopolymer is sodium alginate, the fully soluble salt is calcium chloride, and the sparingly soluble salt is calcium sulfate. The alginate is present in the amount of at least 0.75% by weight of solution, preferably about 1.5%. The freely soluble salt provides 10 to 15 mM calcium ion, while the sparingly soluble
25 salt is provided as a powder dispersed in the alginate solution in an amount which would supply approximately 8 times more calcium ion if it were completely dissolved. In other words, calcium is supplied as the divalent metal cross-linking agent by two salts, calcium chloride and calcium sulfate, in a ratio of 1 part to 10 parts by weight. In a preferred method of preparing
30 the hydrogel suspension, 9 parts of a 2% alginate solution are mixed with 2

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parts of a cell suspension in a suitable cell suspension medium such as M-199, and subsequently 1 part of the same cell suspension medium containing 1.8% anhydrous calcium chloride with 18% calcium sulfate suspended therein is added to the alginate-cell mixture. The final mixture is mixed
5 thoroughly, taking adequate precautions to avoid damaging the cells, and used for injection into tissue. A rapid increase in viscosity may be expected within 15 minutes of mixing all of the components together. The consistency of this mixture should be sufficient for the mixture to hold its shape against gravity, but will remain injectable at room temperature for at
10 least 24 hours.

A preferred viscosity for injectable application varies by intended use, but is a balance of parameters that include (a) the formulation reservoir (e.g., syringe size, piston diameter, resistance required for proper "touch" or application rate), (b) application device (catheter or needle length, diameter,
15 composition), (c) receiving tissue resistance to division, and (d) type of distribution required (e.g., topical application to bone or organ surface, injection within tissues without extravasation, etc.). In one embodiment, the composition is formulated to form a partially hardened hydrogel within thirty minutes, preferably within 10-15 minutes at room temperature, the
20 consistency of the partially hardened hydrogel having a viscosity of at least 15,000 centipoise at 25°C, or alternatively being fluid enough for injection through a 23-gauge cannula at a rate of about 10 ml/minute without substantially damaging the cells (i.e., viability of the cells reduced by no more than 25%).

25 Typically, cells are suspended in a hydrogel solution and injected directly into a site in a patient, where the hydrogel hardens into a matrix having cells dispersed therein. Alternatively, cells may be suspended in a hydrogel solution which is poured or injected into a rigid or inflatable mold having a desired anatomical shape, then hardened to form a matrix having
30 cells dispersed therein which can be implanted into a patient. After

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implantation, the hydrogel ultimately degrades, leaving only the resulting tissue. Such hydrogel-cell mixtures can be used for a variety of reconstructive procedures, including custom molding of cell implants to reconstruct three dimensional tissue defects, filling pre-inserted inflatable
5 molds or scaffolds, as well as implantation of tissues generally.

Treatment of vesicoureteral reflux and incontinence is described where chondrocytes, preferably autologous chondrocytes, are mixed with a liquid biodegradable biocompatible polymeric material, such as alginate which can be solidified *in vivo*, or other carrier to form a cell suspension.
10 The cell suspension is injected into the area where reflux is occurring, or where a bulking agent is required, in an amount effective to yield cartilage that provides the required control over the passage of urine. The cell suspension contains chondrocytes, harvested, grown to confluence, passaged as needed, and then mixed with a biodegradable liquid polymer such as
15 alginate, a copolymer of guluronic and mannuronic acid, which is designed to solidify at a controlled rate when contacted with calcium salts. The cells are mixed with hydrogel forming compositions and then injected at the desired site where they proliferate and correct the defect.

Alternatively, chondrocyte cells are mixed with a liquid polymeric
20 material, such as alginate which can be solidified *in vivo*, to form a cell suspension, and injected into the area of the defect, in an amount effective to yield an elevation of the bladder mucosa that corrects the defect, for example, which provides the required control over the passage of urine. Again, a biodegradable polymer, embedded with cells, serves as a synthetic
25 substrate for the injectable delivery and maintenance of tissue architecture in humans that satisfies all the requirements for an ideal injectable substance. A biopsy of auricular cartilage can be easily and quickly performed followed by chondrocyte cell processing and endoscopic injection of the autologous cell/polymer suspension for the treatment of reflux incontinence and other
30 defects. Studies show that chondrocyte cells can be easily harvested and

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combined with alginate *in vitro*, the suspension can be easily injected cystoscopically, and the tissue formed is able to correct vesicoureteral reflux without any evidence of obstruction.

The ideal injectable substance for the endoscopic treatment of reflux should be a natural bulking agent which is non-antigenic, non-migratory, and volume stable. Autologous chondrocyte cells seem to fulfill all of these requirements. Since the cells are autologous, this method of treatment does not require FDA approval. The procedure can be performed under 15 minutes, with a short period of a mask anesthetic, in the outpatient unit, without any need for a hospital stay. Neither vesical nor perivesical drainage is required. Since the whole procedure is done endoscopically and the bladder is not entered surgically, there is no postoperative discomfort whatsoever. The patient can return to a normal level of activity almost immediately.

15

EXAMPLES

In order to facilitate a more complete understanding of the invention, a number of Examples are provided below. However, the scope of the invention is not limited to specific embodiments disclosed in these Examples, which are for purposes of illustration only.

20 EXAMPLE 1. Effect of Calcium Sulfate Concentration on Gel Time

This experiment shows the effect of changing calcium sulfate concentrations in the absence of calcium chloride. Calcium sulfate levels were varied from 0.02 g/ml (0.1x) through 0.2 g/ml (1.0x) at 0 °C, 37 °C, and room temperature (23 °C), while keeping other content of the formula unchanged. Sodium alginate, grade UP MVG from Pronova with M/G ratio of 35/65, was prepared as 2% solution in 0.1 molar phosphate buffer, pH=7.4, containing 0.79% sodium chloride. Sodium alginate solution was diluted 1 to 1 with M199 cell culture medium, and calcium sulfate powder was added in amounts ranging from 20 mg/ml to 200 mg/ml. After mixing, sample solutions were held at either ice-bath temperatures (0°C), room

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temperatures (R.T.), or body temperature (37°C). Each sample was tested periodically for gel formation by injecting an aliquot onto a mylar sheet.

The results are shown in Table A. The time for gel formation (indicated as X) and for blocking injection (marked B) is also shown. As
5 can be seen from Table A, in the absence of added calcium chloride, no gel formation was observed on the ice-bath. At room temperature, gel formation occurred between 70 and 100 minutes, and the hydrogel injection was blocked within 20 minutes of that time period. At 37°C, the initial gelation
10 occurred from 32 minutes down to 16 minutes depending on the concentration of calcium sulfate; blockage of injection generally occurred within 15 minutes of initial gelation.

The experiments show that at 0 °C, no gelling (nc) occurred after 120 minutes. At room temperature, gelation started at 90 min for 0.2x and 70 min for 1.0x formula. For body temperature, gelation started at 16 min for
15 1.0x formula, and the gel setting time decreases almost linearly with the decrease in calcium sulfate concentration. Although the calcium sulfate cross-linked, cell-containing hydrogel formula can gel up in 16 minutes at human body temperature, it allows only several minutes for injection, plus it necessitates the addition of heating equipment and a filter attached to the
20 syringe in order to achieve any lot-to-lot consistency in delivery and gelling properties. Both of these requirements will increase the complexity in the formula's clinical application. Temperature plays a very important role in the gel formation speed. From Table A, at $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ level of 0.1 x, i.e. 60 mg in a formulated batch, the gel could set up in 32 minute at body
25 temperature and remain injectable for at least 90 minutes. This best case scenario of CaSO_4 at 37 °C still requires too much time until injection, and is both more complicated and more inconsistent than desired.

Table A. Alginate Gel Set-up and Injectability Experiment

Temp	CaSO ₄	Min	4	8	12	16	18	20	22	24	28	32	36	40	50	60	70	80	90	100	120
C	x0.2g/ ml																				nc
	0.1x																				nc
	0.2x																				nc
0	0.4x																				nc
	0.6x																				nc
	0.8x																				nc
	1.0x																				nc
	0.1x																				nc
	0.2x																	x	x		
R.T.	0.4x																	x	B		
	0.6x																			x ₁ B	
	0.8x																	x ₁ B			
	1.0x																x		B		

[illegible]

Note: x = cross-link, B = block, nc = no cross-link, R.T. = room temperature. Blocked needles might have been caused by CaSO₄ particles and resulting gel formed around these particles. Each sample contains 1.5 ml 2% Sodium Alginate in pH 7.4 buffer, 1.5 ml M199 Medium (no cells), and indicated amounts of CaSO₄ powder. Materials are stored on ice prior to mixing. After mixing, samples are stored at the indicated temperature, and injected at the indicated time onto mylar sheet.

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Example 2. Mixtures of Calcium Chloride and Calcium Sulfate.

In a preferred formulation, sodium alginate is dissolved in pH 7.4 K_2HPO_4 buffer. Cells are supplied in M199 medium and then suspended in the alginate solution. Calcium sulfate powder is stored in a 1.5 ml vial.

- 5 Calcium chloride is dissolved in M199 medium with concentration of 18 mg/ml and stored in another vial. In the application, the $CaCl_2$ solution will be mixed with $CaSO_4$ powder, and then mixed with alginate, taken into syringe and wait for about 15 minutes, the formula is ready to be injected.

- In addition to test tube mixing on a vortex, experiments also were run
 10 which show that syringe mixing of this formula exhibited the same gelling characteristics. Experiments were also performed wherein chondrocyte cells were added into the formula, and it successfully gelled up at 14 minutes at room temperature, but remained injectable for more than two hours. Later experiments show that this formula can maintain its injectability for more
 15 than two weeks. Table 2 lists the components in this formula.

Table 1. Components in Formula

Material	Working Conc.	Amount	Final Conc.
alginate ¹	20 mg/ml	2.25 ml	15 mg/ml
Cells in M199	120 million/ml	0.5 ml	20 million/ml
20 $CaSO_4 \cdot 2H_2O$	powder	45 mg	15 mg/ml
$CaCl_2 \cdot 2H_2O$ ²	18 mg/ml	0.25 ml	1.5 mg/ml

¹alginate used is UP MVG grade, dissolved in K_2HPO_4 buffer

² $CaCl_2$ is dissolved in M199 medium.

25 **EXAMPLE 3. Temperature Effect on Gelling Time**

- The time required for alginate to gel when mixed with calcium-chloride/calcium sulfate salt mixture varies with temperature. Sodium alginate, grade UP MVG from Pronova, was prepared as 2% solution in 0.1 molar phosphate buffer, pH=7.4, containing 0.79% sodium chloride. 2.25
 30 ml of alginate solution was mixed with 0.5 ml of M199 cell culture medium. To this mixture, was added 0.25 ml of cell culture medium containing 18 mg

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calcium chloride per ml and 45 mg suspended calcium sulfate powder. After mixing, each sample was held at the indicated temperature and tested periodically for gel formation. The time at which gel formation was detected in duplicate samples is shown in the accompanying table. At room temperature (20-30°C) gel formed from 13 to 30 minutes. At 37°C, gel formation took 7-8 minutes.

Table 2. Time to Gel at Different Temperatures

	Temperature	Gelling Time
	(C)	(min)
10	50	5, 6
	40	7, 7
	37	7, 8
	35	9, 9
	30	13, 14
15	25	17, 18
	20	28, 30
	15	53, 54
	10	120, 115
	5	240, 240
20	0	120 < t < 19 hr.

EXAMPLE 4. Formation of Injectable Alginate Gel

The sensitivity of the time for gel formation to slight variations in the concentration of various components in the mixture was tested as described below. Solutions of sodium alginate, calcium chloride, and calcium sulfate, were prepared as described as in Example 1 and 2, except that the amount of one or another component was varied by plus or minus 16% of the base

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value. The samples were held at room temperature after mixing, and tested periodically for gel formation.

The time for gel formation and the period during which the consistency remained suitable for injection are shown in Table 3. The amount of each component is indicated in the table as M for the base value, H for 16% above the base value, and L for 16% below the base value. Within the variations of these parameters, the gel time was 18 minutes plus or minus about 15%, indicating little effect of variation in any particular component within this range. Although all of these solutions gelled within about 20 minutes, the consistency of the alginate gel was still acceptable for injection beyond 24 hours later. Samples of this formulation prepared in a similar manner demonstrated suitable injectability properties over one month after formulation.

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Table 3. Time for Gel Formation

	No. of Expts.	Algin-ate	M199	CaSO ₄	CaCl ₂	Gel Time (min)	Inject-ability	Ambient Temp. (°C)
5	4	M	M	M	M	18,18,18,17	> 24 hr	23
	4	M	M	M	<u>H</u>	18,18,17,16	> 24 hr	23
	6	M	M	M	<u>L</u>	20, 20, 19,	> 24 hr	21
						20, 20, 19		
	2	H	H	H	H	18, 19	> 24 hr	22
10	2	L	L	L	L	18, 19	> 24 hr	22
	2	<u>H</u>	M	M	M	19, 19	> 24 hr	22
	2	<u>L</u>	M	M	M	18, 17	> 24 hr	21
	2	M	<u>H</u>	M	M	20, 21	> 24 hr	21.5
	2	M	<u>L</u>	M	M	19, 19	> 24 hr	21.5
	2	M	M	<u>H</u>	M	17, 18	> 24 hr	22
	2	M	M	<u>L</u>	M	21, 20	> 24 hr	22
15	H (+16%)	2.6 ml	0.58 ml	0.052 g	0.29 ml			
	M	2.25	0.5	0.045	0.25			
	L (-16%)	1.9	0.42	0.038	0.21			

Notes: 1. Alginate concentration is 2% by weight/volume

2. CaCl₂ concentration is 18mg/ml in M199 medium

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Example 5. Comparison of CaSO_4 powder to $\text{CaCl}_2/\text{CaSO}_4$ mixtures

This experiment was performed to quantitatively characterize the gel and gelling process of two formulations and qualitatively determine the strength of the final gels. During the experiments described above, gelation and the time needed to set gel were determined by visual observation. By using a viscometer, the viscosity change when alginate starts to gel up can be directly measured, and also the relative strength of the gels can be qualitatively determined.

Alginate gelled by CaSO_4 powder was prepared as described for Example 1, and alginate gelled by a mixture of $\text{CaCl}_2/\text{CaSO}_4$ was prepared as described for Example 2. The $\text{CaCl}_2/\text{CaSO}_4$ samples were prepared with syringe mixing, and CaSO_4 powder samples were prepared with vortex mixing method. Syringe mixing allows a very simple procedure that is easy to perform and yields a very consistent product. It also permits the use of approved biomedical devices, and creates a closed system to segregate product from environmental exposure. Viscosity changes along the gelling course of the two systems at temperatures of 5°C , 25°C and 37°C were measured and the result are plotted in Figure 1 through Figure 3.

At room temperature, the $\text{CaCl}_2/\text{CaSO}_4$ sample gelled to an injectable set point¹ at 13 minutes, while the CaSO_4 -only sample did not set the gel until minute 56. The viscosity of the $\text{CaCl}_2/\text{CaSO}_4$ sample before gelling is

¹ Injectable set point: consistency of gel that can be stacked without collapsing.

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about 665 cP, and CaSO_4 -only sample is 202 cP; the former is approximately 3.3 times as thick as that of the latter. When the alginate starts gelling, its viscosity increases abruptly and reaches very high values (Fig. 1), going from 1100 cP to 30,000 cP in 2 minutes. After gelling, the viscosity of the

5 $\text{CaCl}_2/\text{CaSO}_4$ sample is about 2 times that of CaSO_4 -only sample (about 40,000 cP vs. 20,000 CP).

Gelation of both formulations are accelerated at body temperature. $\text{CaCl}_2/\text{CaSO}_4$ sample starts gelation at 4 minutes, and CaSO_4 -only sample gels up at 13 minutes. The viscosities before gelling are about 520 and 135

10 cP respectively for $\text{CaCl}_2/\text{CaSO}_4$ sample and CaSO_4 -only sample at 37°C. The lower initial viscosity is due to the higher temperature. After gelation, the viscosity of the $\text{CaCl}_2/\text{CaSO}_4$ sample could reach 16,600 cP which is approximately 10 times higher than that of CaSO_4 -only formula (Fig. 2), indicating that mixing CaCl_2 and CaSO_4 produces a stronger gel which may

15 permit a better tissue separation in implantation application.

Gelation is dramatically retarded at lower temperatures. Similarly, the $\text{CaCl}_2/\text{CaSO}_4$ sample is more viscous than the CaSO_4 -only formula at all time points at 5°C (Fig. 3).

Compared to the CaSO_4 -only formula, using a mixture of CaCl_2 and

20 CaSO_4 gives: 1) 3 to 4 times higher initial viscosity; 2) much shorter time to reach injectable consistency; 3) higher viscosity after gelation, thus a better tissue separation.

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For purposes of clarity of understanding, the foregoing invention has been described in some detail by way of illustration and example in conjunction with specific embodiments, although other aspects, advantages and modifications will be apparent to those skilled in the art to which the invention pertains. The foregoing description and examples are intended to illustrate, but not limit the scope of the invention. Modifications of the above-described modes for carrying out the invention that are apparent to persons of skill in medicine, reconstructive surgery, cell culture, and/or related fields are intended to be within the scope of the invention, which is limited only by the appended claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

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CLAIMS:

1. A method for treating anatomical defects in an animal in need of treatment thereof comprising
5 preparing a suspension of cells uniformly dispersed in a biodegradable, biocompatible hydrogel solution which is partially hardened, and
implanting the suspension into the animal at the site of the defect, wherein the hydrogel solution fully hardens after implantation.
- 10 2. The method of claim 1, wherein said suspension of dissociated cells in the partially hardened hydrogel is suitable for injection through a 23-gauge cannula at a rate of 10 ml/min. without substantially damaging the cells.
3. The method of claim 1, wherein the defect is vesicoureteral
15 reflux.
4. The method of claim 1, wherein the patient has incontinence.

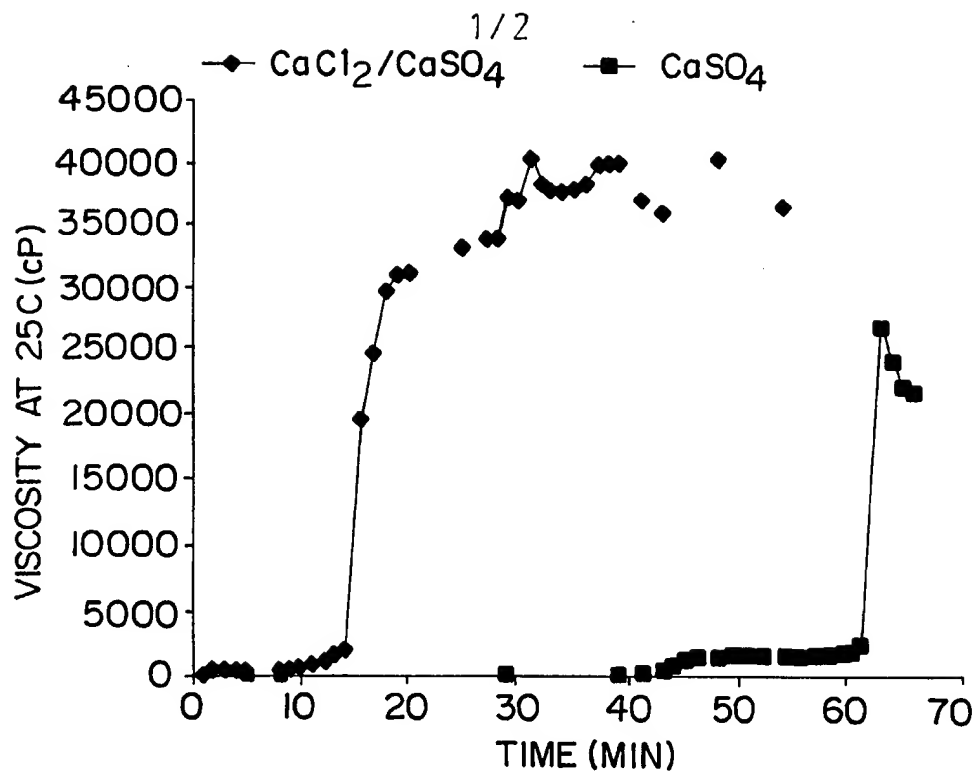


FIG. 1

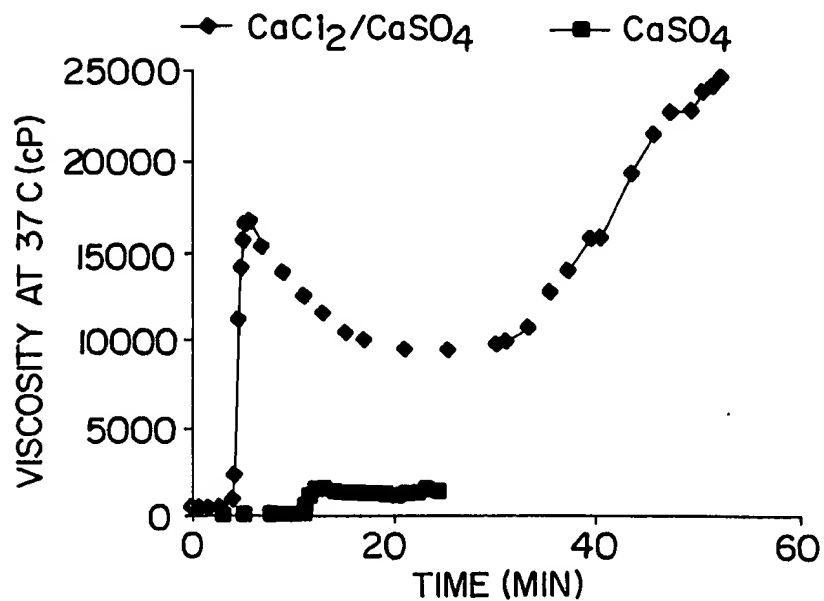


FIG. 2

2 / 2

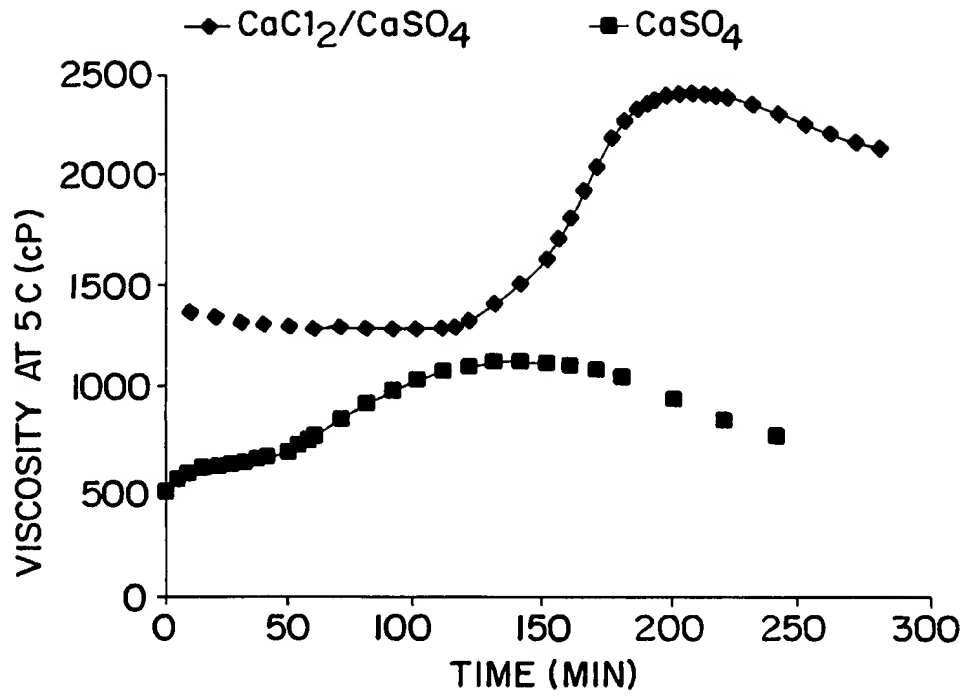


FIG. 3

Elaborate demonstrative exhibits are more likely to impede than help an oral argument. The most effective demonstrative exhibits tend to be a handout or binder containing the demonstrative exhibits. The pages of each demonstrative exhibit should be numbered to facilitate identification of the exhibits during the hearing, particularly if the hearing is recorded.

¶ 124.5 Transcription of oral argument

Transcription of oral argument is strongly encouraged. The party requesting transcription must arrange for the transcription and pay the costs. Parties are encouraged to share the costs.

When an argument is to be transcribed, the party should notify a Trial Division paralegal assistant (571-272-9797) as soon as possible, but at least one business day prior to oral argument, so that arrangements may be made in the hearing room for the reporter.

The transcriber must use a stenography machine, but may also use a tape recording device as a backup. Microphones at individuals' locations are not authorized.

¶ 125 Rehearing of decisions

¶ 125.1 Time for filing

A request for rehearing must be filed within fourteen (14) days of the decision, Bd.R. 125(c)(1), *unless* a judgment accompanies the decision in which case the request must be filed within thirty (30) days of the judgment, Bd.R. 127(d).

¶ 125.2 Format for request for rehearing

A request for rehearing is, in form, a miscellaneous motion, but no prior conference call is required. The argument responsive to the decision must be made with particularity in the following manner:

On page __, lines __-__, the opinion states __. The opinion is believed to have overlooked [or misapprehended] __. This point was set forth in __ Motion [or Opposition or Reply] __ at page __, lines __-__.

The request must include as an appendix an evidence list setting forth a list (in numerical order by exhibit number) of each exhibit that the party believes was overlooked or misapprehended.

¶ 125.3 Number of requests

A party may file no more than one request for rehearing per motion decision.

¶ 125.4 New evidence on rehearing

Evidence not already of record at the time of the decision will not be admitted absent a showing of excusable neglect for the belated submission. Bd.R. 4(a).

¶ 126 Settlement discussions required

¶ 126.1 Last-named party initiates

The party named last in the caption set in the declaration is responsible for—

- initiating any settlement discussions,
- initially drafting any document, and
- initiating any conference call required by this paragraph.